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The goals of this project are: a) To isolate marine Caulobacter bacteria and characterize them by physiological and genetic criteria, b) analyze the adhesive holdfast organelles of various isolates via isolation and chemical analysis and cloning of holdfast related genes, and c) develop and characterize the capabilities of marine Caulobacters for molecular genetic manipulation. This includes methods of introduction of genes and plasmids and the development of expression vectors.

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Progress Report on Contract N00014-87-G-0127
June 28, 1988

Principle Investigator John Smit

Contractor University of British Columbia

Contact Title Characterization of Biofouling Marine Caulobacters and their Adhesive Holdfast

Start Date 1 March 1987

Research Objectives a) To isolate marine Caulobacter bacteria and characterize them, b) analyze the adhesive holdfast organelle, c) Develop and characterize the capabilities of marine Caulobacters for molecular genetic manipulation.

Progress

On the distribution and characteristics of Caulobacters in the marine environment

There is no reliable way to precisely quantitate either the absolute abundance of Caulobacters in an environment or relative fraction of the total marine bacterial population. This is owing to the fact that Caulobacters are difficult to identify in rapid screening methods, the fact that they are caught up in complex aggregations of bacteria that cannot be adequately dissociated for individual culturing and the long standing knowledge that no more than 50% of the bacteria noted in the ocean can subsequently be grown by traditional bacteriological culturing methods. Nevertheless, during the year we continued a qualitative effort to isolate Caulobacters from approximately 40 sites, most along the Western coast ranging from San Diego, California to Vancouver. We were always able to isolate Caulobacters from a seawater sample and conclude that they are ubiquitous in at least shallow nearshore regions of the marine environment and that most, if not all, are part of the group of marine bacteria that can be cultured readily. In efforts to characterize the isolates, we learned that approximately half can grow in anaerobic conditions (contrary to the established literature), about 1/3 have significant resistance to Hg^{2+} via inducible mercury reductases, only a few harbor native plasmids.

On the composition of the adhesive produced by Caulobacters

A major area of investigation is the analysis of the adhesive holdfast of marine and freshwater Caulobacters. The holdfast is essentially a small amount of a very sticky polysaccharide located at the end of a stalk on the bacterium which allows attachment to a variety of surfaces. During the year we completed a preliminary study of the composition of the holdfasts of marine and freshwater Caulobacters, using lectin binding, inhibition of lectin binding by characterized saccharides and by evaluating sensitivity to a variety of glycolytic and proteolytic enzymes. We concluded that all marine Caulobacters have N-acetylglucosamine in their holdfast and nothing else detectable by lectins (although surely there are other substituents). Moreover, the N-acetylglucosamine is present as stretches of 3 or more units. We have found no enzyme that has any effect on the holdfasts, including chitinases, which normally cleave polymers of N-acetyl glucosamine. This is perhaps understandable from an ecological standpoint, since chitinases are very commonly produced by other marine bacteria. It will be intriguing to discover the biochemical basis for this resistance to degradation. Freshwater Caulobacters have more variability in holdfast composition. The holdfast of some strains bound lectins other than N-acetylglucosamine-specific ones. Even those that bound N-acetylglucosamine-specific lectins

were distinguishable from marine Caulobacters in that the holdfasts were sensitive to chitinases.

We have also been developing methods to isolate the polysaccharide in quantity for in-depth chemical and structural analysis. During the year we concentrated on a procedure based on the observation that holdfasts bind very tightly to colloidal gold particles. Once bound, the holdfast is no longer sticky and the complex can be isolated readily with CsCl density centrifugation, relying on the high density imparted by the gold binding. The method developed worked reasonably well and will allow subsequent chemical analysis and production of antisera during 1988.

On the adhesive properties of the holdfast. We also initiated studies aimed at discerning what types of surfaces to which the Caulobacters will attach. This was approached by the preparation of glass surfaces covalently modified with a variety of chemical substituents (provided by Dan Rittschoff, Duke University Marine Labs), resulting in surfaces ranging from highly charged to very hydrophobic. A quantitative attachment assay was developed. Among the things learned was that Caulobacters will attach to virtually all surfaces at some frequency, but appear to prefer substrates in approximately the middle of a hydrophobicity/hydrophilicity scale. Freshwater Caulobacters attach better to very hydrophobic surfaces than do marine Caulobacters. By growing marine strains that tolerate low ionic strength media in a freshwater medium, we learned that the salts in seawater are apparently responsible for lowered adhesiveness to hydrophobic surfaces. Of practical significance was the finding that dimethyldichlorosilane treated glass (ie classical "silanizing") was reasonably effective in discouraging attachment, a convenience for many future experiments, especially holdfast isolation procedures.

On the genes that specify the holdfast structure During the year we began an effort to isolate holdfast-related genes from selected freshwater and marine strains. This required development of a rapid screening technique for detecting holdfast defective strains. Two methods were devised, one involves attaching colonies to cellulose acetate plastic and staining those bacteria that remain after a vigorous washing (in a search for those that fail to remain attached). The other procedure involves attachment of colonies to glass fiber filters and subsequent staining with Congo Red dye, which appears to be reasonably specific to the holdfast of marine Caulobacters.

We also initiated steps to prepare transposon-mutagenized populations of Caulobacters. The use of a transposon insertion allows rapid detection and isolation of the specific gene affected. We prepared a library of 15000 independent insertion in a freshwater Caulobacter strain and have isolated 70 holdfast-defective mutants. We have begun a similar process in two marine strains.

On the development of molecular genetic capabilities of marine Caulobacters. Very little was known about the capabilities for genetic manipulation of this group of marine bacteria. Such information is needed, not only for the isolation and characterization of holdfast genes, but also to proceed with a number of other biotechnology-related ideas related to positive uses of biofilm forming bacteria. During the year we completed studies aimed at discerning which strains of marine Caulobacters were most suitable for conjugal transfer of plasmids (an essential method needed to introduce foreign genes), what antibiotic resistance markers can be expressed in these bacteria, and at what concentrations (necessary for cloning experiments) and what gene promoters can be recognized in selected strains (necessary for the expression of foreign genes). We are also determining which transposons can be used to generate mutants in marine Caulobacters, ie, which will efficiently transpose to random genomic locations.

The general conclusions are encouraging; we have found no significant difficulties in selecting suitable strains for most standard genetic manipulations, most antibiotics can be used, the promoter for a freshwater Caulobacter gene (a highly expressed surface protein) is recognized by many of the marine Caulobacters and can be used for expression vector construction and transposons Tn5 and Tn7 at least transpose in several of our marine strains.

Work Plan

Holdfast analysis

We will concentrate on the isolation and chemical characterization of the holdfast from two marine strains and two freshwater Caulobacters, each of which showed differences in chemical composition (by lectin-binding analysis) and adhesiveness. We shall be engaged in gas-liquid chromatography/mass spectroscopy of the isolated material to learn the monosaccharide composition and linkages of these holdfasts. We shall also begin development of additional purification procedures; the method involving binding to colloidal gold has some drawbacks for future analyses, such as NMR.

We shall use the transposon-generated mutants to isolate holdfast genes from the freshwater Caulobacters. We will learn whether the genes are disperse in the genome or clustered as an operon(s). We shall repeat the transposon mutagenesis and gene cloning process in several marine Caulobacters. If the freshwater genes are homologous to the marine counterparts, these will be used as probes to speed up the cloning process.

Molecular Genetics of Marine Caulobacters

We shall demonstrate the utility of various transposons in various marine Caulobacters, as indicated above. This and other information is leading us toward a decision as to which marine strain to concentrate on for extensive genetic analysis. In collaboration with Bert Ely, University of S. Carolina, we will begin the construction of a physical genetic map for a selected strain, using pulsed field electrophoresis techniques. We shall also investigate the electroporation method for the introduction of plasmids in to marine Caulobacters; we have already been successful with freshwater Caulobacters and expect the method to work. We shall also extend our efforts to generate a stable plasmid vector for marine strains, based on a cryptic plasmid from on of the marine strains. The goal is to clone the replication origin and regions necessary for stability and thereby produce a vector for foreign gene cloning that will be nontransferable and stable without selection.

Publications (relevant to the ONR funding)

Abstracts

•Nivens, D.E. , A. Tunlid, M.J. Franklin, J. Smit, and D. White, 1988 Infrared monitoring of interactions between Caulobacter species and solid surfaces. Abstracts of the 88th Annual Meeting, American Society for Microbiology, 1988.

Publications

•Smit, J. 1987 "Caulobacters in the marine environment" *in* Marine Biodeterioration: Advanced Techniques Applicable to the Indian Ocean. Edited by the American Institute of Biological Sciences, Oxford and IBH Publishing, New Delhi, India.

•Anast, N. and J. Smit. 1988 Isolation, characterization of marine Caulobacters and assessing the potential for genetic experimentation. Appl. Environ. Micro. 54: 809-817.

•Merker, R. M. and J. Smit 1988 Analysis of the adhesive holdfast of marine and freshwater Caulobacters., in press, Appl. Environ. Micro -August issue.

•Fisher, J., J. Smit and N. Agabian. Transcriptional analysis of the major surface array gene of Caulobacter crescentus. in press, J. Bacteriol.

Manuscripts currently in preparation
•Merker, R. M., D. Rittschoff and J. Smit. Analysis of the attachment of marine and freshwater Caulobacters to surfaces using chemically defined substrates. for Appl. Environ. Microbiol.

•Nivens, D.E. , A. Tunlid, M.J. Franklin, J. Smit, and D. White. 1988 Infrared monitoring of interactions between Caulobacter species and solid surfaces.
Merker, R.M. and J. Smit. Isolation and preliminary chemical analysis of the adhesive holdfast of marine and freshwater Caulobacters.

Training Activities
Two graduate students are currently supported by this contract. During the past year one postdoctoral was supported by the contract.

Awards
•Appointment of John Smit as an associate member of the Oceanography Department, University of British Columbia, July 1987.
•Appointment of John Smit to the Education Committee of the Canadian Society of Microbiologists, June 1988.

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